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Ligand: A Novel Gene Therapy Strategy for Prostate  
Cancer

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13. ABSTRACT ( <i>Maximum 200 Words</i> )  <p><u>Introduction:</u> Fas is a transmembrane receptor which mediates apoptosis when transactivated by FasL. Earlier studies illustrated the ability of IFN-<math>\gamma</math> to increase Fas expression on RM-1 mouse prostate cancer cells. To kill the remaining Fas positive cells, the potential of combining adenovirus-mediated expression of FasL (Ad.FasL) with IFN-<math>\gamma</math> <i>in vitro</i> and Ad.IL-12 to stimulate host production of IFN-<math>\gamma</math> <i>in vivo</i> was addressed.</p> <p><u>Results:</u> Alone Ad.FasL resulted in killing equal to the degree of transduction. The addition of IFN-<math>\gamma</math> enhanced killing to maintain maximal cell kill with only 6% of cells expressing FasL. In vivo with a constant Ad.IL-12 dose and increasing doses of Ad.FasL, tumors were 25% smaller at lower doses tumors, increasing to 66% smaller at <math>1 \times 10^9</math> pfu. In a dose controlled experiment with equal doses of each vector (<math>1 \times 10^9</math> pfu), individually Ad.FasL and Ad.IL-12 resulted in 27+/-10% and 54+/-4% smaller tumors with combination therapy resulting tumors which were 72+/-6% smaller than controls. However, the combination of Ad.FasL and Ad.mIL-12 resulted in a worse survival than for Ad.IL-12 alone. However, the injection of Ad.mIL-12 into the normal contralateral prostate combined with the injection of the tumor by Ad.FasL resulted in superior survival.</p> <p><u>Conclusions:</u> These studies validate the concept of exploiting Fas upregulation for fasL transactivation. The ability to eradicate tumors is currently being addressed by sequential injection of Ad.FasL.</p>				
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## Introduction

Fas is a potential target expressed on the cell surface of many tumor cells. The binding of its ligand, FasL, initiates an intracellular cascade resulting in apoptosis.<sup>i</sup> This pathway was first identified within the lymphoid system where it is thought to act within the homeostatic mechanisms of T cells and other effectors. It is unknown why many tumors, including prostate, express Fas, but its use as a potential therapeutic target has generated interest. The idea of harnessing the Fas/FasL pathway as a vehicle for killing prostate cancer arose from studies looking at the growth suppression observed following injection of an adenovirus expressing IL12 (Ad.IL12) in an orthotopic mouse model of prostate cancer (RM-1).<sup>ii,iii</sup> Studies have noted significant local and systemic growth suppressive activities following a single intra-tumoral injection of Ad.mIL-12. Studies linked the ability of IL-12 to direct host T cells and NK cells to produce IFN $\gamma$  which in-turn increased tumor expression of Fas from 30 to 60-70%.<sup>iv</sup> Exposure of RM-1 cells to IFN- $\gamma$  in vitro resulted in a dose-dependent killing as great as 30%. This effect could subsequently be blocked by adding anti-FasL IgG. Indicating interaction of constitutively low FasL expression (15%) and upregulated Fas expression. In-vivo blockade of Fas/FasL resulted in a modest reduction in local tumor killing. Interestingly, immunohistochemical studies showed that most of the surviving tumor cells were Fas+ and thus targets for FasL transactivation. Immunohistochemical studies have shown that human prostate cancers express Fas<sup>v,vi,vii</sup>. Established human prostate cancer cell lines have also been found to express Fas. Though many are generally resistant to agonist antibody Fas transactivation<sup>viii,ix,x,xi</sup> the exposure of normally Fas resistant human prostate cancer cells (LNCaP and PC3) to IFN- $\gamma$  for 24-48 hours restored Fas sensitivity to soluble FasL in vitro.<sup>xii</sup> Therefore the concept of adding vector mediated FasL to kill Fas+ cells was explored. Logical extension of this then led to combination of vector mediated IL-12 to stimulate host production of IFN- $\gamma$  to up-regulate Fas/FasL to potentiate the tumoricidal effect of Fas and FasL.

## Body

### 1) Construction of an Adenovirus Expressing FasL.

The first priority for this proposal was the construction of an adenovirus expressing FasL. PCR amplification of FasL was performed, followed by ligation into an adenovirus backbone driven by the Rous Sarcoma Virus promoter. The pAd.RSV-FasL was co-

transfected with pBHG10 in 293 cells by the calcium phosphate precipitation method to yield Ad.FasL. Large-scale production of vector was achieved following expansion in 293 cells and double cesium gradient ultracentrifugation purification. As discussed in the original application the problem to overcome with this process was the inhibition of apoptosis of the 293 cells used to plaque recombinants and expand the vector, as they express high levels of FasL. We proposed using the caspase inhibitor, Z-VAD-FMK (20uM Enzyme System Products, Livermore, CA) to avoid this problem. However, we were unable to to successfully plaque a sFasL, even with 100uM Z-VAD-FMK, presumably due its more potent ability. Therefore, for the time being we have proceeded with the membrane bound FasL due to these technical difficulties and the high likelihood that the soluble FasL construct will be excessively toxic.

## 2) in Vitro Activity of Ad.FasL

An intentionally low dose of IFN $\gamma$  was used to minimize cytokine-mediated killing and to maximize the chance of witnessing cooperative actions. However, the addition of Ad. $\beta$ -Gal resulted in a killing of ~30% (Figure 1). To further understand this phenomenon we are exploring the possibility of Fas/FasL interactions via Fas upregulation from IFN- $\gamma$  and FasL upregulation due to virus infection. Alone Ad.FasL demonstrated dose dependent killing in a fashion consistent with the transduction efficiency of RM-1 cells. Killing at the MOI (multiplicity of infection, ie the number of virus particles per cell) 25 which correlates to 25% was 30-35%. The combination of Ad.FasL with IFN $\gamma$  induced and maintained maximum cell kill to between 60-70% at 4-fold lower vector dose, indicating synergistic killing (Fig 1, IFN $\gamma$  vs. Combination therapy  $p=0.035$ , Mann-Whitney). Killing of 60% of the cells with only 6% of cells expressing FasL would indicate the presence of a bystander effect as hypothesized.

## 3)In vivo Gene Therapy:

Dose escalations of Ad.FasL performed in mice given a standard dose of Ad.mIL12 ( $1 \times 10^9$  pfu) demonstrated a significant suppression of tumor growth at Ad.FasL doses greater than  $1 \times 10^9$  pfu. (Fig 2,  $1 \times 10^8$  pfu vs.  $1 \times 10^9$  pfu,  $p=0.003$ , t-test) No additional benefit was evident from viral dosing greater than  $1 \times 10^9$  pfu. In fact higher Ad.FasL doses actually trended for larger tumors but this was not found to be statistically significant ( $p = 0.16$ , t-test).

Dose control experiments were performed to determine the relative effectiveness of the individual and the combined treatments with Ad.IL12 and Ad.FasL. Vector inoculation into tumor of C57BL6 mice with established prostatic tumors (RM-I) with Ad.FasL alone at dose of  $1 \times 10^9$  resulted in a 27% (+/-11%) decrease in tumor size from controls (Fig 3,  $p=0.04$ , t-test). Ad.mIL12 inoculation alone (dose  $1 \times 10^{10}$  pfu) into tumor resulted in a 66% (+/-7%) decrease in tumor size ( $p=0.001$ , t-test). Combined viral therapy with both Ad.mIL12 and Ad.FasL resulted in a 72% (+/-6%) decrease in tumor size (Ad.IL12 vs. Combination therapy,  $p=0.04$ , t-test). In addition to fully exploit remaining Fas+ cells an additional injection of FasL was performed on the third day post-Ad.FasL+Ad.mIL-12 injection. However, no improvement in growth suppression over a single Ad.FasL injection was noted.

In a survival study both a single and double injection of Ad.FasL improved survival over that of controls but surprisingly was inferior to Ad.mIL-12 (Fig 4, Ad.mIL-12: 22.8+/-8 days vs combo Tx: 19.3+/-6 days,  $p=0.0134$ , mantel-Cox). We reasoned that perhaps combination therapy was inferior to due to the killing of Ad.mIL-12 transduced cells, thereby reducing both IL-12 and IFN- $\gamma$  levels. This in turn may reduce the other abilities of IL-12 in this model, induction of cytotoxic neutrophils, T cells, and NK cells, and the upregulation of Fas through a reduction of IFN- $\gamma$ . To test this hypothesis we injected Ad.mIL-12 into the contralateral lobe of the prostate which did not harbor a tumor with the injection of the tumor by Ad.FasL as before. In this assay as would be predicted injection of the contralateral lobe reduced the survival enhancing abilities of cytokine therapy, but when combined with intratumoral injection with Ad.FasL resulted in significantly enhanced tumor growth suppression (Fig 5, Ad.mIL-12 intratumoral versus Ad.mIL-12 contralateral + intratumoral FasL,  $p=0.027$  Mantel-Cox). This study would indicate that indeed the best strategy will be an injection of IL-12 away from the site of Ad.FasL injection to ensure high serum levels of IFN- $\gamma$  combined with repetitive injections of Ad.FasL.

## Key Research Accomplishments

- production of replication incompetent adenovirus expressing FasL
- demonstration of synergistic killing in vitro
- enhancement of growth suppression in vivo

## Reportable Outcomes:

### Abstracts:

1. CANFIELD S, HASSEN WA, ATKINSON G, AND HALL SJ. TUMOR CELL EXPRESSION OF FAS AS A TARGET FOR PROSTATE CANCER THERAPY. J UROL 163, ABSTRACT 161, 2000.
2. SELLECK WA, CANFIELD SE, HASSEN WA, AND HALL SJ. IL-12 AND FASL GENE THERAPY RESULT IN ENHANCED GROWTH SUPPRESSION IN AN ORTHOTOPIC MODEL OF PROSTATE CANCER. ACCEPTED FOR AUA, 2001.

## CONCLUSIONS

Studies thus far have supported the hypothesis that the combination of Ad.mIL-12 and Ad.FasL would be beneficial in controlling locally aggressive prostate cancers. The finding that co-injection would result in a worsening of activity in longer-term studies was surprising. However, it appears the solution to that problem has been solved. At present the effectiveness of sequential injections of Ad.FasL to eradicate tumors is under study. With this knowledge the focus of study will shift to human cell lines through an understanding of the mechanisms of IFN- $\gamma$  reversal of resistance and design of a specific vector.

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- <sup>i</sup> Hug, H.: Fas-mediated apoptosis in tumor formation and defense. *Biol Chem*, 378: 1405, 1997.
- <sup>ii</sup> Hall, S.J., Mutchnik, S.E., Chen, S-H, Woo, S.L.C., and Thompson, T.C.: Adenoviral mediated herpes simplex virus thymidine kinase gene transduction and gancyclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer: *Int J Cancer*, 70:183, 1997
- <sup>iii</sup> Hall, S.J., Sanford, M.A., Atkinson, G. and Chen, S-H.: Induction of potent anti-tumor natural killer cell Ad<sub>HSV-tk</sub> and gancyclovir therapy on an orthotopic mouse model of prostate cancer. *Cancer Res*, 58: 3221, \_\_\_\_
- <sup>iv</sup> Canfield, S.J., Hassen, W., Atkinson, G., and Hall, S.J.: Tumor cell expression of Fas as a target for prostate cancer gene therapy (abstract). *J Urol*, 163: 37, 2000
- <sup>vv</sup> Rokhin, O.W., Bishop, G.A., Hostager, B.S., Waldschmidt, T.J., Sidoenko, S.P., Pavlov, N., Kiefer, M.C., Umanski, S.R., -----, R.A. and Cohen, M.B.: Fas-mediated apoptosis in human prostate cancer cell lines by Fas. *Int J. Cancer*. 67:709, 1996
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- <sup>viii</sup> Liu Q.Y., M.A. Rubin, C. Omene, S. Lederman, and C.A. Stein. 1998. Fas ligand is constitutively secreted by prostate cancer cells in vitro. *Clin. Cancer Res*. 4: 1803.
- <sup>ix</sup> Rokhlin O.W., G.A. Bishop, B.S. Hostager, T.J. Waldschmidt, S.P. Sidorenko, N. Pavloff, M.C. Kiefer, S.R. Umansky, R.A. Glover, and M.B. Cohen. 1997. Fas-mediated apoptosis in human prostatic carcinoma cell lines. *Cancer Res*. 57: 1758.
- <sup>x</sup> Uslu R., N. Borsellino, P. Frost, H. Garban, C.P. Ng, Y. Mizutani, A. Belldgrun, and B. Bonavida. 1997. Chemosensitization of human prostate carcinoma cell lines to anti-Fas-mediated cytotoxicity and apoptosis. *Clin. Cancer Res*. 3: 963.
- <sup>xi</sup> Costa-Pereira A.P. and T.G. Cotter. 1999. Camptothecin sensitizes androgen-independent prostate cancer cells to anti-Fas-induced apoptosis. *Br. J. Cancer*. 80: 371.
- <sup>xii</sup> Canfield, S.J., Hassen, W., Atkinson, G., and Hall, S.J.: Tumor cell expression of Fas as a target for prostate cancer gene therapy (abstract). *J Urol*, 163: 37, 2000



## Figure Legends

1. Ad.FasL in vitro. Plated RM-1 cells were exposed to IFN- $\gamma$  (25u/ml) or PBS. Twenty-four hours later each group was subdivided to receive escalating doses of Ad.FasL or Ad. $\beta$ -gal for 2 hours. The following day viable cells as per trypan blue exclusion were counted. Each data point represents the average of triplicate wells  $\pm$  SD.

2. Escalating Doses of Ad.FasL in vivo. RM-1 cells were injected into the right dorsolateral prostate. Six days later established tumors were randomized to receive an injection of Ad.mIL-12 ( $1 \times 10^9$  pfu) and escalating doses of Ad.FasL. Eight days later all mice were sacrificed and the wet weight of excised tumors recorded. Each bar represents the average weight  $\pm$  SD.

3. Dose Controlled study of Ad.mIL-12 and Ad.FasL. RM-1 cells were injected into the right dorsolateral prostate. Six days later established tumors were randomized to receive an injection of Ad. $\beta$ gal ( $2 \times 10^9$  pfu), Ad.mIL-12 ( $1 \times 10^9$  pfu), Ad.FasL ( $1 \times 10^9$  pfu) or Ad.mIL-12 + Ad.FasL ( $1 \times 10^9$  pfu each). Some combination mice received a second injection of Ad.FasL on the 3<sup>rd</sup> day post vector injection. Eight days later all mice were sacrificed and the wet weight of excised tumors recorded. Each bar represents the average weight  $\pm$  SD.

4. Survival Study with Ad.mIL-12 and Ad.FasL. RM-1 cells were injected into the right dorsolateral prostate. Six days later established tumors were randomized to receive an injection of Ad. $\beta$ gal ( $2 \times 10^9$  pfu), Ad.mIL-12 ( $1 \times 10^9$  pfu), Ad.FasL ( $1 \times 10^9$  pfu) or Ad.mIL-12 + Ad.FasL ( $1 \times 10^9$  pfu each). Some combination mice received a second injection of Ad.FasL on the 3<sup>rd</sup> day post vector injection. Endpoint death or sacrifice when mice appeared stressed by weight loss, lethargy or ruffled fur.

5. Survival study Comparing Contralateral injection of Ad.mIL-12 with Intratumoral Ad.FasL. RM-1 cells were injected into the right dorsolateral prostate. Six days later established tumors were randomized to receive an injection of Ad.FasL ( $1 \times 10^9$  pfu), Ad.mIL-12 ( $1 \times 10^9$  pfu), or Ad.mIL-12 + Ad.FasL ( $1 \times 10^9$  pfu each). Ad.mIL-12 was injected either into the tumor or in the contralateral, normal prostate. Endpoint death or sacrifice when mice appeared stressed by weight loss, lethargy or ruffled fur.

Figure 1

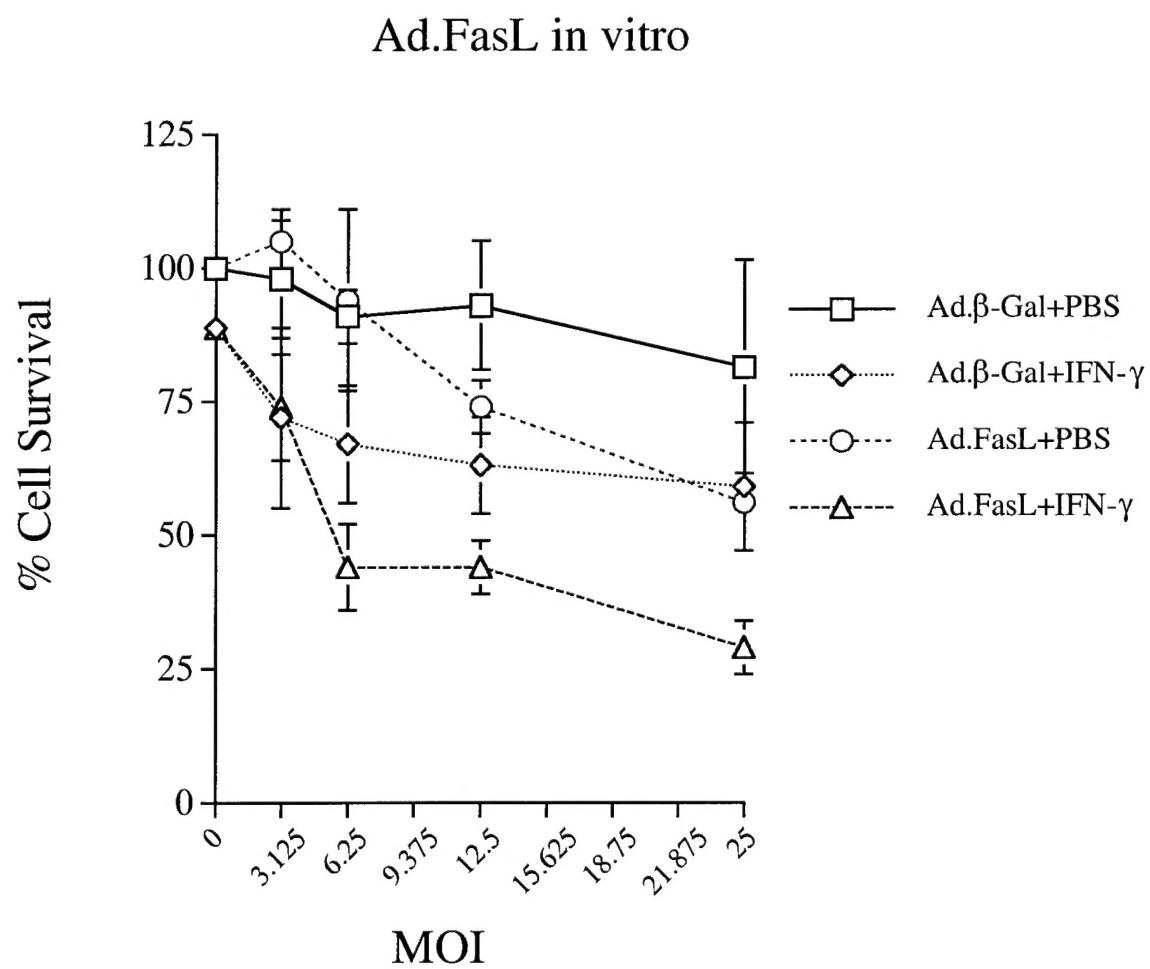


Figure 2

Constant Ad.mIL-12 Dose  
Versus  
Escalating Doses of Ad.FasL

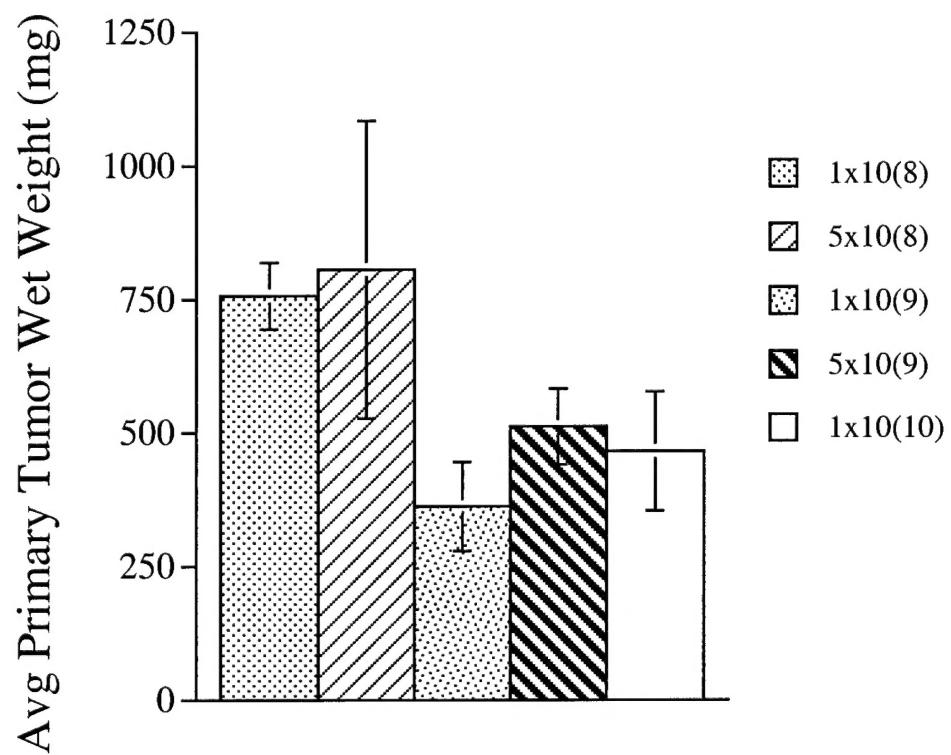


Figure 3

Dose Controlled Study  
of  
Ad.FasL + Ad.mIL-12

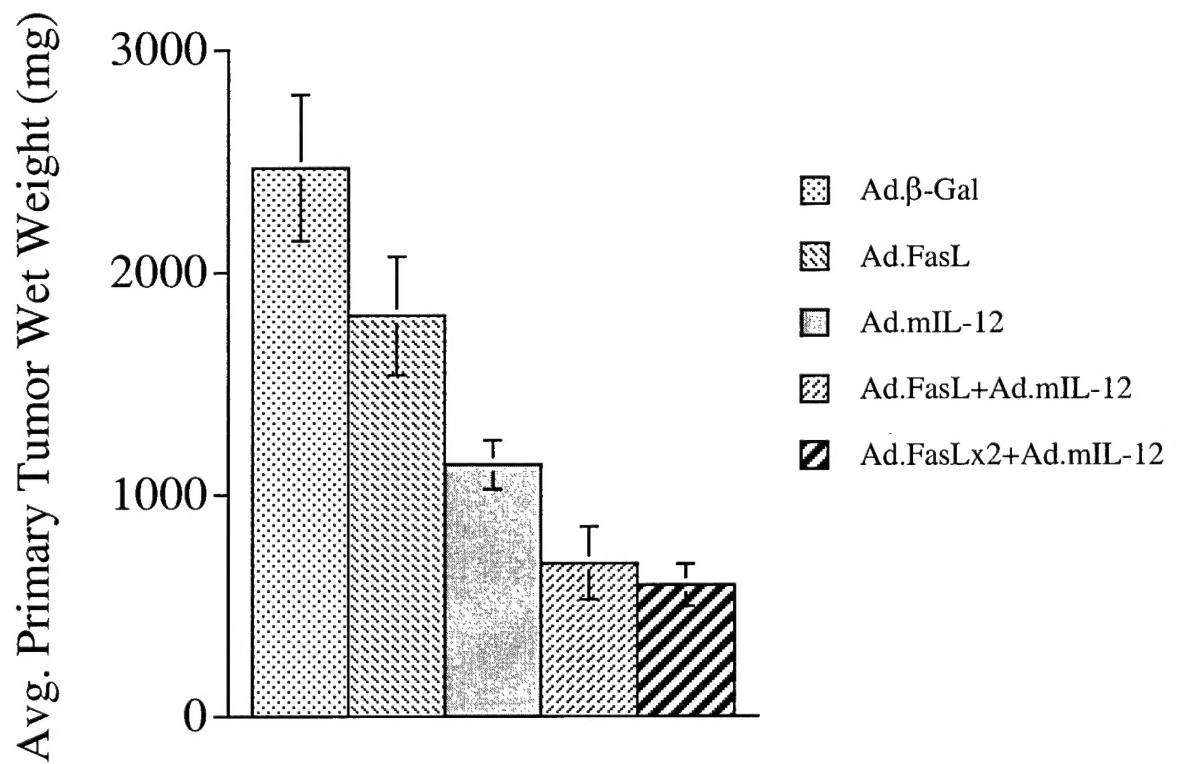


Figure 4

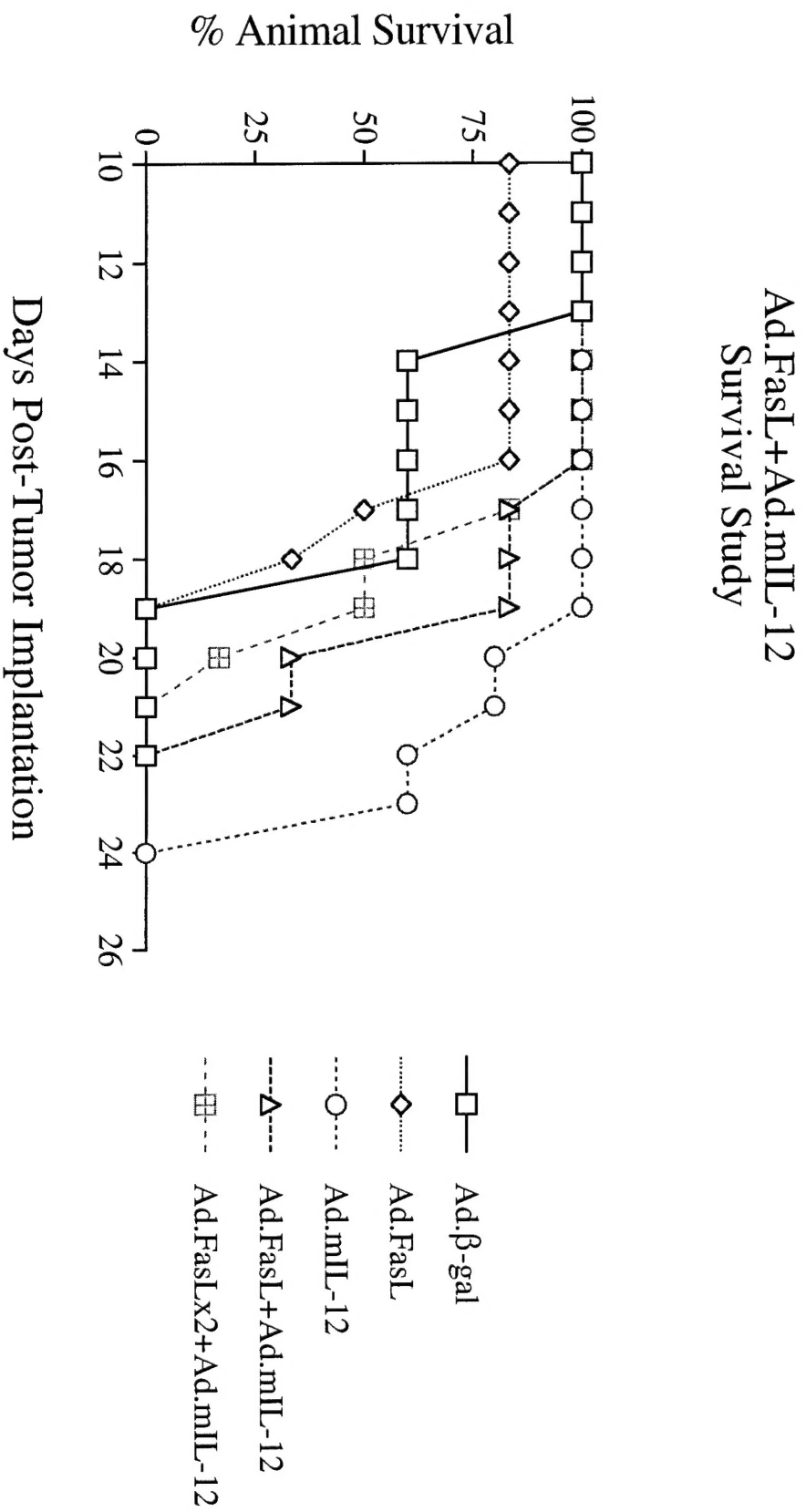


Figure 5

# Variation of Vector Injection Site Effect on Survival

